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THE SIGNIFICANCE OF CHANGES IN HIGH MOBILITY GROUP-1 PROTEIN mRNA EXPRESSION IN RATS AFTER THERMAL INJURY

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ABSTRACT—There has been a widespread impression that tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) mediate the toxicity of high doses of lipopolysaccharide (LPS, endotoxin) and are key factors in septic shock. However, the clinical efficacy of treatment with antagonists of TNF-α and IL-1β is still controversial, suggesting that mediators other than TNF-α and IL-1β might contribute causally to endotoxin-induced death. Recent studies implicated high mobility group-1 (HMG-1) protein as a late mediator of endotoxin lethality in mice. However, the role of HMG-1 in mediating multiple organ damage-associating trauma has not been studied. This study was designed to investigate changes in HMG-1 gene expression in vital organs, and its potential role in mediating multiple organ damage following major burns. Wistar rats were subjected to a 35 percent full-thickness thermal injury, and randomly divided into three groups as follows: normal controls (n = 7), thermal injury (n = 24), and recombinant bactericidal/permeability-increasing protein ($rBPI_{21}$) treatment (n = 12). Tissue samples from liver and lungs were collected to measure tissue endotoxin levels and HMG-1 mRNA expression. In addition, blood samples were obtained for measurement of organ function parameters. Our data demonstrated a significant increase in HMG-1 gene expression in tissues at 24 h postburn, which remained markedly elevated up to 72 h after thermal injury ($P < 0.05$–0.01). Treatment with $rBPI_{21}$ could significantly decrease tissue HMG-1 mRNA expression in the liver and lung ($P < 0.01$). In addition, there were high positive correlations between hepatic HMG-1 mRNA and serum aminoleucine transferase (ALT) and aspartate aminotransferase (AST) levels, and also between pulmonary HMG-1 mRNA and myeloperoxidase activities ($P < 0.05$–0.01). Taken together, these findings indicate that thermal injury *per se* can markedly enhance HMG-1 gene expression in various organs. Up-regulation of HMG-1 expression may be involved in the pathogenesis of endogenous endotoxin-mediated multiple organ damage secondary to major burns.

KEYWORDS—Burns, high-mobility group proteins, gene expression, recombinant bactericidal/permeability-increasing protein, endotoxin translocation, multiple organ dysfunction syndrome

INTRODUCTION

Lipopolysaccharide (LPS, endotoxin), an integral component of the gram-negative bacterial cell membrane, has strong pro-inflammatory properties and can activate multiple inflammatory cascades (1). Recent studies implicated high mobility group-1 (HMG-1) protein as a late mediator of endotoxin lethality in mice (2). HMG-1 was found to be released by cultured macrophages more than 8 h after stimulation with endotoxin. Meanwhile, mice showed increased serum levels of HMG-1 from 8 to 32 h subsequent to endotoxin challenge. Moreover, recombinant HMG-1 induced an endotoxemia-like state in mice, and HMG-1 *per se* produced lethality when directly administered in large amounts. The above data provide support for the contention that HMG-1 is a late component of the host response to endotoxemia. However, the role of HMG-1 in mediating multiple organ damage associated with trauma has not been studied.

A naturally occurring compound with potent anti-endotoxin activity that has recently been studied as a potential therapeutic agent in the treatment of sepsis is bactericidal/permeability-increasing protein (BPI). BPI has a high-affinity binding domain for the lipid-A domain of endotoxin (3). In addition to avidly binding free endotoxin and suppressing endotoxin-mediated cellular activation, BPI also has potent antibacterial properties specific for gram-negative bacteria (4). An increasing body of circumstantial evidence has been accumulated that BPI and its recombinant N-terminal fragments could provide protection against challenge with bacteria or purified bacterial endotoxin in several animal models (5, 6) and, more recently, in human clinical trials (7, 8). Moreover, Yao et al (9) reported that $rBPI_{21}$ (a 21 kD amino-terminal fragment of BPI) might be a useful therapeutic agent against endogenous bacteria or endotoxin related disorders in severe hemorrhagic shock. Therefore, the aim of this study was to investigate the relationship between endotoxin translocation and tissue HMG-1 mRNA expression after burns by treatment with $rBPI_{21}$, and to define the potential role of HMG-1 in the pathogenesis of multiple organ damage.

MATERIALS AND METHODS

Animals and thermal injury

Male Wistar rats (weight range 250–300 g), purchased from the Laboratory Animal Center, Beijing, China, were used for the study. The animals were housed in separate cages in a temperature-controlled room with alternating 12-h light-dark cycles, and were allowed to acclimatize for at least 7 days before being used. All animals had free access to water, but were fasted overnight prior to the experiment. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40mg/kg body weight), the hair removed from the animal's dorsum, and a 35% total body surface area full-thickness thermal injury was created by immersing dorsal skin in a 100°C water bath for 12 sec. All animals received Ringer's solution (50ml/kg body weight) after thermal injury for resuscitation, and the burn wounds

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were treated with 1% silver sulfadiazine everyday as a prophylactic measure against wound infection. After thermal injury, all animals had free access to water and food, eating and drinking in a similar matter. All experimental manipulations were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Burn Institute, Postgraduate Medical College, Beijing.

Experimental design

43 scalded animals were randomly divided into three groups as follows: (1) normal control group (n = 7); (2) thermal injury group (n = 24), being further divided into 12, 24, 48, 72-h postburn groups; (3) recombinant bactericidal/permeability-increasing protein ($rBPI_{21}$) treatment group (n = 12), being further divided into 12, 24-h postburn groups. In a pilot experiment, main parameters determined in the current study in normal animals were found to be highly constant, without marked change over time. Thus, time course control was not included in this study. Animals received a 2mg/kg dosage of $rBPI_{21}$ (XOMA Corp., Berkeley, CA, BPI group) or the control protein (albumin, Sigma Chemical, St. Louis, MO, burn group) in the same dose in an intravenous bolus at 30 min and 4 h, respectively, after thermal injury. Under anesthesia, systemic blood samples were obtained. Then the animals were killed with an overdose of pentobarbital sodium and tissue specimens were taken from liver and lungs.

Tissue endotoxin measurement

Tissue specimens from liver and lungs were aseptically removed, and were homogenized in 3-fold volume pyrogen-free saline on ice. Then, the homogenate were stored at −20°C until analysis. The tissue endotoxin levels were measured by the chromogenic Limulus Amebocyte Lysate (LAL) assay. The procedure was based on the LS-1 kit (Seikagaku Corp., Tokyo, Japan) protocol modified by perchloric acid (PCA) treatment of samples to remove nonspecific activators or inhibitors of the lysate (10). The endotoxin concentration in endotoxin units per gram of organ tissue was calculated from a standard curve derived from assay of standards. The limit of detection of this method was 0.01 EU/mL.

RNA extraction and reverse-transcription polymerase chain reaction

Tissue specimens from liver and lungs were removed, then were stored in liquid nitrogen until analysis. Extraction of total tissue RNA was performed with guanidine isothiocyanate according to the method described by Chromczynski and Sacchi (11). First-strand cDNA was synthesized using oligo-dT primer and the AMV reverse transcriptase (Promega Corp., USA).

The generated cDNA was then added to the reaction mixture with a final concentration 0.2 μmol/L of specific primers. The PCR mix contained a final concentration of 1 × PCR buffer, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP and 0.7 U/25 μl *Taq* polymerase (Promega Corp., U.S.). For the amplification of the desired cDNA, the following gene-specific primers were used: HMG-1: 5′-CCCGCGGATCCTCGAGGGAAGGATGGGCAAAGGAGATCCTA-3′ and 5′–CCCGCAAGCTTATTCATCATCATCATCTTCT-3′ (2); glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-TCCCTCAAGATTGTCAGCAA-3′ and 5′-AGATCCACAACGGATACATT-3′ (12). After a 5-min initial melting step at 97°C, 30 cycles of PCR were carried out (1 min, 94°C denaturation; 1 min, 56°C annealing; and 1 min, 72°C extension). The final cycle was then followed by a 10-min soak at 72°C. Since expression of house keeping gene GAPDH mRNA in liver or lungs remained constant after thermal injury in the present study, and no markedly age-related alterations and interindividual variation in the expression of GAPDH mRNA were observed, GAPDH was used as internal controls for standardization of PCR product. DNA was amplified simultaneously for each organ, using the primer HMG-1 and the primer of GAPDH.

PCR products and molecular weight markers were subjected to electrophoresis on 2% agarose gels in TAE buffers at 5 V/cm for 1 h and visualized by means of ethidium bromide staining. The number of PCR cycles was selected for HMG-1 so that most of the ethidium bromide-stained amplified DNA products were between barely detectable and below saturation. The gel then was photographed, and the negative scanned with a densitometer (Pharmacia Corp., Sweden). The ratios of HMG-1/GAPDH signals were calculated for each sample. Each experiment included a negative control (sample RNA that had not been subjected to reverse transcription). This sample did not yield a PCR product, confirming the absence of extraneous genomic DNA of PCR product contaminating the samples.

Myeloperoxidase (MPO) assay

Lung tissue was homogenized in a 9-fold volume 0.02 mol/L potassium phosphate buffer, pH 7.4, and centrifuged for 30 min at 35,000 rpm, 4°C (13). MPO activity per gram wet lung (gwl) was calculated by the following formula: Myeloperoxidase activity (units/gwl) = $(\Delta A_{460}) \pm (13.5)$/ lung weight (g), where ΔA_{460} is the change in absorbance of 460 nm from 30 to 90 sec after the initiation of the reaction. The coefficient 13.5 was empirically determined such that 1 unit MPO activity is the amount of enzyme that will reduce 1 μmol peroxide/min.

Liver function measurement

Systemic blood samples were collected, and serum was prepared by centrifugation for 10 min at 2,000 rpm. Then, the samples were stored at −20°C until analysis. Serum aminoleucine transferase (ALT) and aspartate aminotransferase (AST) levels were determined with a biochemical autoanalyzer (Hitachi Corp., Japan).

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical evaluation of the continuous data was performed by one-way analysis of variance (ANOVA), followed by Dunnett's *t* test for between-group comparisons. Correlations between variables were tested by Spearman's correlation coefficients. These statistical analyses were done using the statistical package SAS 6.04. The level of significance was considered to be $P < 0.05$.

RESULTS

HMG-1 mRNA expression in local tissues

To examine the tissue-specific expression of HMG-1 gene *in vivo*, and to evaluate the time course of HMG-1 mRNA induction, rats were subjected to thermal injury. Then tissue samples (liver and lungs) were removed at various time points, and total RNA was analyzed for HMG-1 mRNA by RT-PCR. It was shown that normal animals constitutively expressed HMG-1 mRNA in the liver and lungs (Fig. 1). During the early postburn phase, HMG-1 mRNA levels were not significantly different between normal and burned animals ($P < 0.05$). As shown in Figure 1A, a significant increase in hepatic HMG-1 mRNA levels was observed at 24 h postburn (1.95-fold of preburn value), which remained markedly elevated up to 72 h following thermal insult. HMG-1 mRNA expression in lungs showed a similar tendency (Fig. 1B).

Inhibitory effect of $rBPI_{21}$ on HMG-1 mRNA expression in tissues

It was found that endotoxin levels in liver and lungs increased markedly after thermal injury, peaking at 12 h and keeping a high level till 72 h. To further investigate the potential role of endotoxin translocation in mediating HMG-1 induction, the influence of $rBPI_{21}$ treatment (2 mg/kg) on postburn HMG-1 mRNA expression was observed. The results showed that the early increase in hepatic endotoxin levels (17.337 ± 3.687 EU/g) was significantly inhibited by $rBPI_{21}$ treatment (4.768 ± 0.687 EU/g, $P < 0.05$). Meanwhile, the $rBPI_{21}$-treated group had a statistically significant decrease in the hepatic HMG-1 mRNA expression compared with the controls (Fig. 2A, $P < 0.01$). Likewise, when pulmonary levels of endotoxin in rats treated with $rBPI_{21}$ decreased, levels of HMG-1 mRNA in lungs in the treatment group also declined to the baseline values at 12 h (Fig. 2B).

The relationship between HMG-1 and liver function parameters

Serum ALT and AST levels markedly increased after scald injury, reaching a maximum between 12 and 24 h (4–5 folds, $P < 0.01$), remaining higher than baseline levels at 72 h ($P < 0.05$–0.01). Hepatic HMG-1 mRNA and serum ALT levels were positive correlated at 24 h ($r = 0.87$, $P < 0.01$), 48 h ($r = 0.84$, $P < 0.05$), 72 h ($r = 0.75$, $P < 0.01$) postburn (Table 1). Similar results were also obtained between hepatic HMG-1 mRNA and serum AST levels ($P < 0.01$).

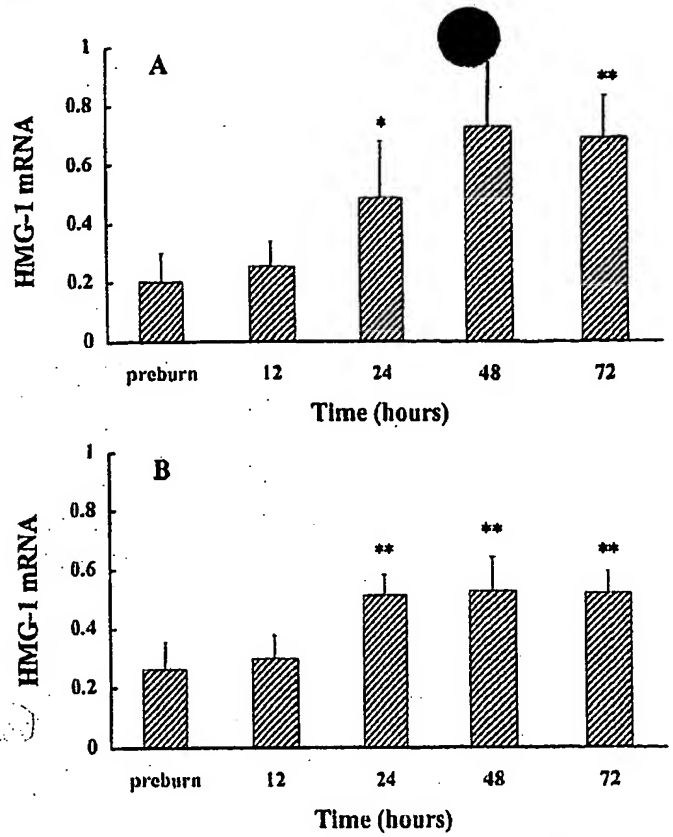

FIG. 1. Semiquantitive RT-PCR analysis of HMG-1 mRNA in (A) liver and (B) lungs after thermal injury. Values are reported as the ratio of HMG-1 to GAPDH signals. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to the normal control group (shown as "the preburn").

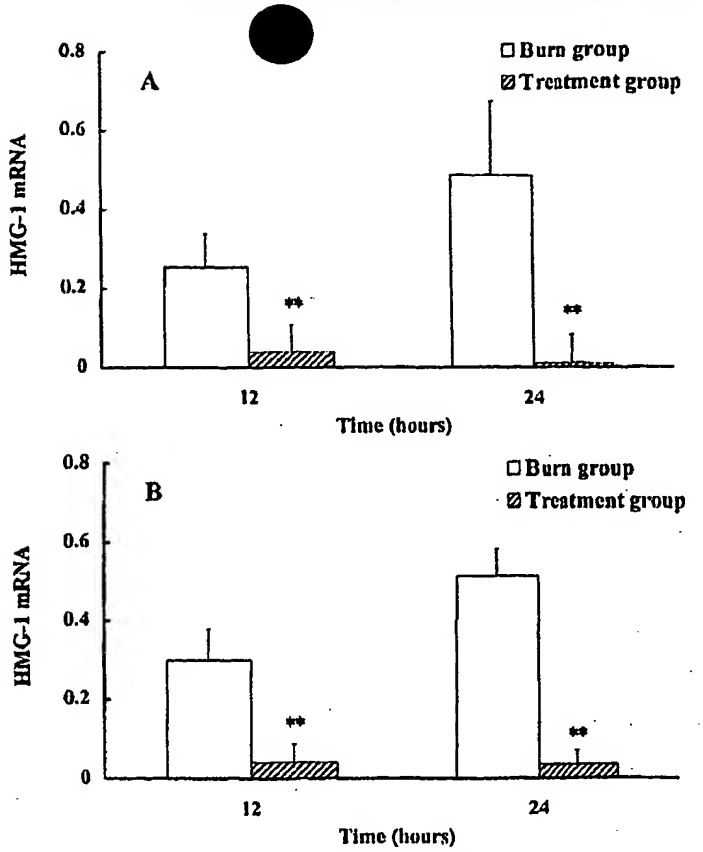

FIG. 2. The effect of $rBPI_{21}$ on HMG-1 mRNA expression in (A) liver and (B) lungs after thermal injury. Animals in the treatment group received a 2mg/kg dosage of $rBPI_{21}$ in an intravenous bolus at 30 min and 4 h after thermal injury. HMG-1 mRNA in tissues was measured by semiquantitive RT-PCR. Values are reported as the ratio of HMG-1 to GAPDH signals. $^{**}P < 0.01$ as compared to the burned group.

The relationship between HMG-1 and MPO activity

In current study, pulmonary neutrophil sequestration was expressed by MPO activity. The results showed MPO activity increased markedly after burns, peaking at 12 h ($P < 0.01$), and maintaining a high level till 72 h. It was noted significant correlations between pulmonary HMG-1 mRNA and MPO activities at 24 h ($r = 0.92$, $P < 0.01$) and 48 h ($r = 0.88$, $P < 0.05$) following burns.

DISCUSSION

The high-mobility group (HMG) proteins are among the most abundant and ubiquitous non-histone chromosomal proteins, which are classified into three families: the HMG-1/2, HMG-I(Y) and HMG-14/17 families (14). Although the structure of the proteins is well defined, their cellular function is not fully understood. Most of the data suggest that these proteins serve as "architectural" elements in chromatin. Common properties of HMG-1 proteins include interaction with the minor groove of the DNA helix (15, 16), binding to irregular DNA structures (17, 18), and the capacity to modulate DNA structure by bending (19). Furthermore, numerous lines of evidence suggest that HMG-1 proteins participate in activating a number of regulators of gene expression (20, 21). It was only recently that the biological role of HMG-1 protein was also ascribed as an important factor in a mouse model of endotoxic shock (2). Herein, we investigated the potential role of HMG-1 in pathophysiologic alterations induced by endogenous endotoxin after major burns.

The expression of HMG-1 during the cell cycle and differentiation has been extensively studied (22). However, much less is known about the regulation of HMG-1 under non-growth-related conditions, *in vitro* or *in vivo*, such as the ability of trauma to regulate HMG-1 expression in local tissues. To our knowledge, the present study revealed for the first time that thermal injury induced a delayed increase of HMG-1 mRNA expression in various tissues. During the early postburn phase, HMG-1 mRNA levels were not significantly different between normal and burned animals. A dramatic increase in HMG-1 mRNA expression was observed 24 h postburn, and it maintained markedly elevated up to 72 h following thermal injury. The induction of HMG-1 mRNA by thermal injury might be due to an increase in gene transcription and/or in mRNA stability. Similarly, Ombrellino el al. reported that serum HMG-1 concentrations increased within the first 24 h after the onset of hemorrhagic shock, and remained high for 72 h (23). It has also been reported that serum HMG-1 levels were significantly increased in patients with sepsis (2). These data, in conjunction with our results, indicate that *in vivo* HMG-1 levels could be upregulated by a variety of insults. Moreover, the time course of the response demonstrates that HMG-1 upregulation is a late and persistent event and quite differs from the kinetics of previously described early inflammatory mediators, such as TNF-α and IL-1β.

Recent studies using in vitro systems demonstrated that LPS or mediators induced by LPS could induce the release of HMG-1 (2, 24). Since our previous studies have shown that burn injury *per se* could result in translocation of gut-derived

TABLE 1. Correlations between tissue HMG-1 gene expression and organ function parameters

	Time (hours)			
	12	24	48	72
Hepatic HMG-1 mRNA/ALT				
r	0.32851	0.87185	0.84172	0.75167
P	0.3881	0.0022	0.0088	0.0315
Hepatic HMG-1 mRNA/AST				
r	0.32191	0.88989	0.84370	0.79621
P	0.3982	0.0013	0.0085	0.0188
Pulmonary HMG-1 mRNA/MPO				
r	0.49945	0.92379	0.87908	0.44946
P	0.2538	0.0010	0.0495	0.3117

endotoxin, which was accumulated in various tissues (25), we examined the influence of endotoxin translocated from gut on tissue HMG-1 mRNA expression after thermal injury. It was found in the present study, that when endotoxin levels in organs were attenuated by $rBPI_{21}$ treatment, tissue HMG-1 mRNA expression also significantly decreased, suggesting that local endotoxin may play an important role in up-regulating HMG-1 expression after acute insults. These observations are in agreement with a recent study showing that serum HMG-1 concentrations were significantly increased after systemic LPS exposure (2). In addition to LPS, *in vitro* studies with cultured pituicytes and macrophages have shown that TNF-α and IL-1β could also lead to the release of HMG-1 in a time- and dose-dependent manner (2, 24). Moreover, this release was synergistically enhanced by interferon gamma. Since these early, acute cytokines might function as upstream regulations of HMG-1 release, the upregulation of HMG-1 after thermal injury might be due to the direct and/or indirect modulation of endotoxin. In addition, HMG-1 has been suggested to be associated with cell proliferation and differentiation as stated above (22). In this respect, compensatory regeneration of cell death induced by major burns might also account for the elevation in HMG-1 mRNA levels shown here.

We had demonstrated elsewhere that serum ALT levels were significantly reduced when endotoxin levels in hepatic tissues were decreased markedly by $rBPI_{21}$ treatment (26). Additionally, there were highly positive correlations between pulmonary endotoxin levels and MPO activities (25). These data strongly suggested that endotoxin accumulated in local sites might be involved in mediating multiple organ damage secondary to major burns. However, definite molecular mechanisms of endotoxin in the pathogenesis of septic shock and organ failure have not been clearly elucidated. In a recent study, HMG-1 has been implicated as an important mediator in a mouse model of endotoxic shock (2). More importantly, systemic administration of HMG-1 was lethal to mice. Since the HMG-1 gene was overexpressed dramatically postburn as stated above, we became interested in the potential role of HMG-1 in the pathogenesis of endotoxin-mediated multiple organ damage associated with thermal injury. The results presented here showed that hepatic HMG-1 mRNA were positively correlated with serum ALT and AST levels at 24, 48 and 72 h postburn. Meanwhile, we found significant correlation between pulmonary HMG-1 mRNA and MPO activities at 24 and 48 h. All these data implied that HMG-1 might be involved in the development of organ damage following thermal injury. In addition, the elevation in HMG-1 expression might also represent compensation for tissue destruction. Unfortunately, the lack of anti-rat HMG-1 antibody deprived us of the opportunity to further define the etiologic role of HMG-1 in the pathogenesis of local and systemic tissue injury associated with endotoxin translocation. Strong supportive evidence comes from a recent experimental study, however, demonstrated that administration of anti-HMG-1 antibody protected mice against lethal endotoxemia even when the delivery was delayed for 2 h after the LPS challenge (2).

In summary, we demonstrated here that thermal injury *per se* could markedly enhance HMG-1 gene expression in different organs. Up-regulation of HMG-1 expression may be involved in the pathogenesis of endotoxin-mediated multiple organ damage secondary to major burns. The results of the present study suggests that either physiological or pharmacological intervention of HMG-1 expression, which appears late in the course of sepsis might be a promising adjunct in the treatment of sepsis.

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